Acute Myeloid Leukemia Immunophenotyping by Flow Cytometric Analysis

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KEYWORDS

• Flow cytometry • Acute myeloid leukemia • Mixed phenotype acute leukemia
• Minimal residual disease

KEY POINTS

- Immunophenotyping by flow cytometry plays a critical role in the diagnosis and classification of AML allowing for blast identification, lineage assignment, and immunophenotypic characterization.
- Flow cytometry can provide useful clues to underlying genetics, reliably distinguish AML from other precursor neoplasms that may be in the differential diagnosis, and provide insight to prognosis.
- AML MRD assessment has been demonstrated to be a particularly powerful tool in AML prognostication.

INTRODUCTION

Over the past 20 years, immunophenotyping by flow cytometry has become an essential laboratory tool in the diagnosis and classification of several hematologic malignancies. The role of this technology in evaluating acute leukemia and lymphoma is particularly well established and widely implemented.[1,2] In acute myeloid leukemia (AML), flow cytometry is crucial for detection of leukemic blasts, blast lineage assignment, and identification of aberrant immunophenotypic features that allow distinction of abnormal blast populations from normal progenitors.

With the elucidation of the antigen expression pattern of a given hematopoietic cell lineage during normal maturation,[3–5] assignment of individual cells to a specific lineage and stage of maturation is achieved with high confidence using a unique combination of markers. Leukemic blasts frequently show immunophenotypic deviation

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from the antigenic expression pattern seen on normal hematopoietic progenitors of similar lineage and maturation stage. This basic principle allows for the recognition of leukemic blasts, determination of the immunophenotype, and subsequent classification in AML.

GENERAL APPROACH TO GATING

Population Identification

Specimens typically assessed by flow cytometry for involvement by AML include peripheral blood and bone marrow. In these specimens, the neoplastic blasts are often present in a background of normal hematopoietic progenitors. CD45 versus side scatter (SSC) gating is an initial strategy for blast identification in many clinical laboratories and provides a useful starting point to distinguish major hematopoietic cell populations (Fig. 1).[6,7] Based on the different intensity of CD45 expression and SSC on the hematopoietic lineages, this method allows for reliable identification of mature lymphocytes, monocytes, maturing granulocytes, myeloid blasts, and lymphoid blasts (hematogones). Although CD45 versus SSC gating is useful to guide initial assessment and allows tracking of a population of interest between tubes, the populations discriminated by this strategy are often not pure and additional lineage-specific markers must be used for definitive population identification.

Panel for Immunophenotyping

The expanded myeloid blasts in AML are often first recognized in a CD45 versus SSC defined "blast gate" with decreased CD45 expression and intermediate SSC.[4] However, several other populations fall within "blast gate" in addition to blasts, including basophils, plasmacytoid dendritic cells, hypogranular myeloid cells, and early monocytic cells. Therefore, additional markers are required to differentiate blasts from other populations in the blast gate.

The antibodies included in a panel used for the diagnosis of AML should be capable of confirming the immaturity of the suspected blast population, assigning the lineage, and delineating the aberrant antigenic expression pattern that distinguishes neoplastic blasts from normal progenitors. For myeloid blasts, a combination of CD34 and CD117 can usually be used to define immaturity. Accurate lineage assignment is a critical component in the diagnosis of acute leukemia. For this reason, the Bethesda International Consensus Conference recommends that the initial assessment of a new acute leukemia includes evaluation of myelomonocytic and lymphoid markers.[8] Myeloid lineage is suggested by expression of antigens including CD13, CD15, CD33, and MPO, whereas monocytic lineage is suggested by expression of antigens including CD4, CD14, bright CD33, and CD64. Assessment of lymphoid markers should be included in the initial evaluation of AML to confirm correct lineage assignment and to evaluate for aberrant expression of a nonlineage marker on an abnormal myeloid blast population. In this regard, a panel for acute leukemia evaluation should include B-cell markers (ie, CD19, CD22, and/or cytoplasmic CD79a) and T-cell markers (ie, CD2, surface and cytoplasmic CD3, CD5, and CD7). Once lineage is confidently assigned, a comprehensive assessment of antigens expressed by the blast population should be made to determine how the aberrant blast immunophenotype differs from that of normal progenitors. Aberrant antigenic expression on the leukemic blasts may include the following[4]: overexpression, underexpression, or loss of an antigen typically expressed by a myeloid blast; asynchronous antigen expression; cross-lineage antigen expression; and abnormally homogeneous antigen expression.

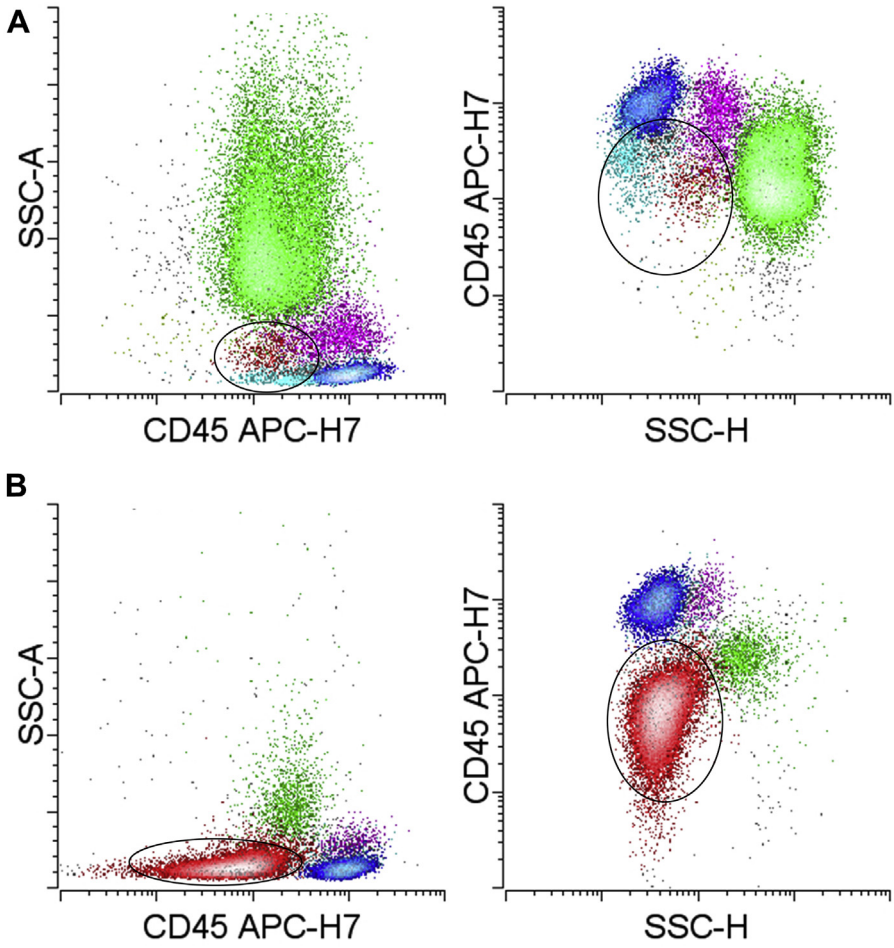

Fig. 1. CD45 versus SSC gating allows separation of various hematopoietic populations. Different laboratories display CD45 versus SSC in different ways with two common approaches being to either show SSC on the y-axis on a linear scale while displaying CD45 on the x-axis on a log scale (*left*), or to show CD45 on the y-axis on a log scale while showing SSC on the x-axis on a log scale (*right*). Both methods provide similar information and work well to separate relevant populations. (*A*) Normal bone marrow. CD45 is expressed with increasing intensity on hematopoietic cells as they mature, whereas SSC is increased on cells with more complex cytoplasm. The combination of these parameters allows separation of lymphocytes (*blue*), hematogones (*aqua*), monocytes (*pink*), granulocytes (*green*), and myeloid blasts (*red*). Using CD45 versus SSC, one can draw a CD45 versus SSC defined blast gate (*oval*). (*B*) Acute myeloid leukemia, not otherwise specified. Flow cytometry of the peripheral blood in this specimen demonstrates an expanded myeloid blast population (*colored red*; 67.3% of the white cells) in the CD45 versus SSC-defined blast gate. Neoplastic myeloid blasts typically have intermediate SSC with lower CD45 expression than mature hematopoietic populations and usually fall into the CD45 versus SSC defined blast gate (*oval*). Neutrophilic cells (*green*) are infrequent in this specimen and show aberrantly decreased SSC.

The panel of antibodies to assess a new acute leukemia in our laboratory is outlined in Table 1. This panel allows for the demonstration of normal maturation patterns of hematopoietic progenitors and thereby allows one to identify and characterize the abnormal immunophenotype of the leukemic blasts.

Blast Enumeration

Flow cytometry may assist in blast enumeration in the peripheral blood; however, enumeration of blasts in the bone marrow is confounded by several factors. Hemodilution artificially decreases the blast percentage and may be especially problematic in the setting of a fibrotic marrow. Additionally, processing of marrow specimens for flow cytometry often removes a subset of erythroid precursors. Excluding erythroid precursors from the denominator artificially increases the blast percentage. Therefore, the morphologic blast count is the most reliable method for blast enumeration.

Other Types of Blast Equivalents

In addition to myeloid blasts, other types of cells may represent blast equivalents for the purposes of diagnosing AML. These include abnormal promyelocytes that may be seen in acute promyelocytic leukemia (APL) and immature monocytic cells (monoblasts/promonocytes) that are seen in acute leukemia with monocytic differentiation. Abnormal promyelocytes have higher SSC than average myeloid blasts and are often excluded from a standard CD45 versus SSC defined blast gate. These are discussed in detail later (see section on AML with recurrent genetic abnormalities). Immature monocytic cells are typically characterized by expression of high levels of CD4, CD33, CD64, and HLA-DR, with intermediate CD15, and variable, often low, CD13. In contrast with more mature monocytic cells, monoblasts and promonocytes usually have low to absent CD14 (Fig. 2).[4,5] It is important to be familiar with the characteristics of the clone of CD14 used in the laboratory because different clones may recognize different proportions of immature monocytic cells.[9] Aberrant CD56 expression may be seen on immature monocytic populations, although this finding is not specific.[10–12]

Table 1
Immunophenotyping panel for the diagnosis of acute myeloid leukemia

Fluorochromes	PB or V450	FITC	PE	PETR	PeCy5, PECy5.5, or PERCP Cy5.5	PECy7	A594	APC	APCA700	APCCy7 or APCH7
Myeloid tube 1	HLA-DR	CD15	CD33	CD19	CD117	CD13	CD38	CD34	CD71	CD45
Myeloid tube 2	HLA-DR	CD64	CD123	CD4	CD14	CD13	CD38	CD34	CD16	CD45
B-cell tube	CD20	Kappa	Lambda		CD5	CD19	CD38	CD10		CD45
T-cell tube	CD8	CD2	CD5	CD34	CD56	CD3	CD4	CD7	CD30	CD45

Abbreviations: A594, Alexa Fluor 594; APC, allophycocyanin; APCA700, Allophycocyanin Alexa Fluor 700; APCCy7, Allophycocyanin cyanine-7; APCH7, Allophycocyanin H7; FITC, fluorescein isothiocyanate; PB, Pacific blue; PE, phycoerythrin; PECy5, Phycoerythrin cyanine-5; PECy5.5, Phycoerythrin cyanine-5.5; PECy7, Phycoerythrin cyanine-7; PerCPCy5.5, Peridinin chlorophyll protein cyanine 5.5; PETR, phycoerythrin Texas red; V450, BD Horizon dye V450.

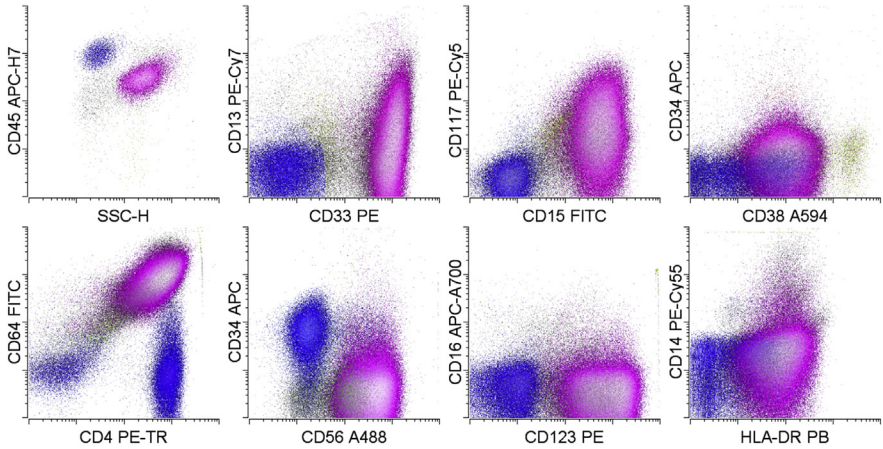

Fig. 2. Acute myeloid leukemia with monocytic differentiation. The monoblasts/promonocytes (*colored pink*; 76.7% of the white cells) in the bone marrow are characterized by increased SSC and expression of CD4, CD13 (heterogeneous), CD15, CD33 (bright), CD38, CD56, CD64, CD117 (heterogeneous), CD123, and HLA-DR without CD14, CD16, or CD34. Background reactive lymphoid cells are shown in *blue*.

Two unusual subtypes of AML include AML with erythroid or megakaryocytic differentiation. Early erythroid precursors (pronormoblasts) are recognized by expression of CD36, CD71, CD117, and CD235a.[13] Megakaryocytic blasts typically show expression of CD41 and/or CD61 on the surface.[14,15] Some studies suggest that use of intracellular CD61 maybe a more sensitive and specific marker.[16] It should be noted that evaluation of CD41 and CD61 can provide some technical challenges because adherence of platelets to blast populations may result in an artifact suggesting megakaryocytic differentiation when it is not present. Adequate washing to remove adherent platelets may be helpful in this regard.[17]

Acute Myeloid Leukemia With Recurrent Genetic Abnormalities

The 2008 World Health Organization (WHO) classification and 2016 Revision divide AML into four major categories: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) therapy-related myeloid neoplasms, and (4) AML, not otherwise specified.[18,19] A subset of AML subtypes show strong correlation between immunophenotype and underlying genetic abnormalities.

AML with t(8;21); *RUNX1-RUNX1T1* is a distinct clinicopathologic entity and displays a characteristic immunophenotypic profile by flow cytometry. The blasts typically show high levels of CD34, HLA-DR, and MPO expression with somewhat weaker expression of CD13 and CD33 (**Fig. 3**).[20–24] Coexpression of B-cell markers is frequently present, and may include CD19, PAX5, and/or cytoplasmic CD79a. CD56 is also expressed in 60% to 80% of cases in AML with t(8;21)[20,25,26]; in some studies, CD56 expression is correlated with an adverse prognosis.[26,27] CD56 is usually positive in t(8;21) AML with KIT-activating mutations, which occur in 20% to 25% of cases and are associated with an inferior outcome.[28,29] It is important to recognize this immunophenotypic pattern to avoid misclassification of this AML subtype as mixed phenotype acute leukemia (MPAL). Of note, AML with t(8;21) may sometimes present with fewer than 20% blasts by morphology. For this reason, the demonstration of coexpression of CD19 and CD56 on a myeloid blast population should raise

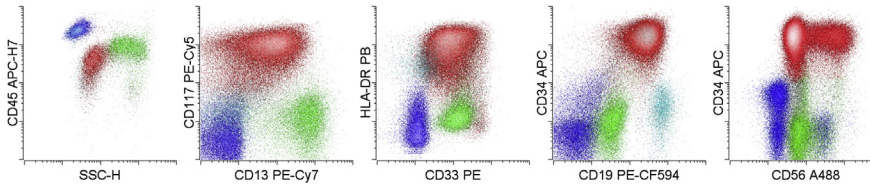

Fig. 3. Acute myeloid leukemia with t(8;21). The leukemic blasts (*colored red*; 36.3% of the white cells) in the bone marrow show expression of CD13 (heterogeneous), CD19, CD33, CD34 (increased), CD45 (decreased), CD56 (subset), CD117 (increased), and HLA-DR. Background reactive lymphoid cells are shown in *blue* and neutrophilic cells are shown in *green*.

the consideration of AML with t(8;21) even in cases with less than 20% blasts by morphology.

In APL with t(15;17), the typical blast equivalent is an abnormal promyelocyte. Characteristically, these blast equivalents have high SSC and express myeloid markers including heterogeneous CD13; low-to-absent CD15; uniformly bright CD33; heterogeneous CD117; and MPO without markers normally expressed at earlier stages of maturation, such as CD34 and HLA-DR (**Fig. 4**).[1,30,31] In the microgranular variant of APL, SSC may be lower and there is frequent variable expression of CD2 and CD34.[32,33] CD56 is expressed in 15% to 20% of APL cases and has been correlated with a poor prognosis.[34–36] Occasionally, basophilic differentiation with expression of basophil-specific marker CD203c is seen in APL during arsenic trioxide therapy.[37]

It is critical to distinguish APL from other AML subtypes lacking expression of CD34 and HLA-DR[38,39] because APL therapy differs from that of other subtypes of AML and typically incorporates all-*trans* retinoic acid.[40–42] Furthermore, APL is frequently associated with a life-threatening disseminated intravascular coagulation, and therefore needs prompt diagnosis and rapid and specific management. The major differential diagnostic considerations include AML with monocytic differentiation (which is most commonly HLA-DR positive allowing distinction of this entity in most cases) and AML with FLT3 and/or NPM1 mutations, which often lack expression of both CD34 and HLA-DR.[43] Although immunophenotype is characteristic, a definitive diagnosis of APL requires confirmatory genetic testing to demonstrate the presence of a PML-RARA fusion.

AML with *KMT2A* (*MLL*) rearrangements commonly shows some elements of monocytic differentiation by morphology and flow cytometry with monoblasts/promonocytes typically predominating as blast equivalents.[44–46] The monocytic blast

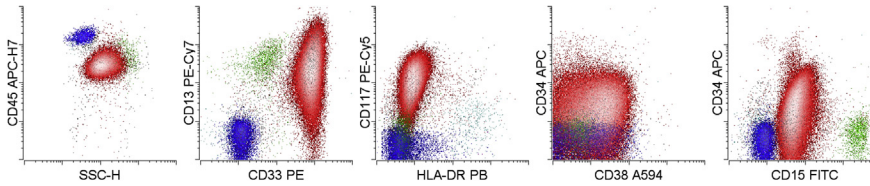

Fig. 4. Acute promyelocytic leukemia. The blast equivalents/abnormal promyelocytes (*colored red*; 90.9% of the white cells) in the bone marrow have increased SSC as compared with typical myeloid blasts. The blast equivalents express CD13 (heterogeneous), CD15 (dim), CD33 (bright), and CD117 without CD34, CD38, or HLA-DR. The dim CD15 expression helps distinguish this abnormal population from normal maturing myeloid cells (at the promyelocyte stage and beyond), which should have a high level of CD15 expression. Background reactive lymphoid cells are shown in *blue* and infrequent background neutrophilic cells are shown in *green*.

equivalents usually express myeloid-associated antigens and monocytic markers including CD4, CD33, CD64, and HLA-DR, whereas expression of CD13, CD14, and CD34 is often low to absent (see **Fig. 2**). Variable levels of CD56 and CD117 have also been described in AML with *KMT2A* (*MLL*) translocations.[45,47]

In addition to recurrent translocations and inversions, gene mutations also occur in AML. These include FLT3 internal tandem duplication, NPM1, and CEBPA mutations, which have been reported in patients with a normal karyotype and have prognostic significance.[48] NPM1 mutations occur in about one-third of adult AML cases and are frequently present in acute myelomonocytic and acute monocytic leukemia.[49,50] AML with NPM1 mutation commonly expresses myeloid-associated antigens and monocytic markers including CD13, CD14, CD33, and MPO, but lacks CD34 expression.[49,51] A subset of AML with cuplike nuclear morphology has been described that expresses CD123 without significant CD34 or HLA-DR, and is highly associated with FLT3 internal tandem duplication and NPM1 mutations (**Fig. 5**).[52]

CLASSIFICATION OF CHALLENGING CASES

Acute Leukemias of Ambiguous Lineage

In the 2008 WHO classification, acute leukemias of ambiguous lineage are defined as leukemias that show no clear evidence of differentiation along a single lineage.[1] They include acute undifferentiated leukemia (AUL) with no lineage-specific antigens on blasts and MPAL with blasts expressing antigens of more than one lineage. Flow cytometry is the preferred method to establish the diagnosis of acute leukemias of ambiguous lineage.

By definition, blasts in AUL do not express significant T, B, or myeloid lineage-specific markers, and lack markers for megakaryocytic, erythroid, and plasmacytoid dendritic cell lineages. MPAL is slightly more frequently than AUL and accounts for 2% to 5% of newly diagnosed AML.[53–57]

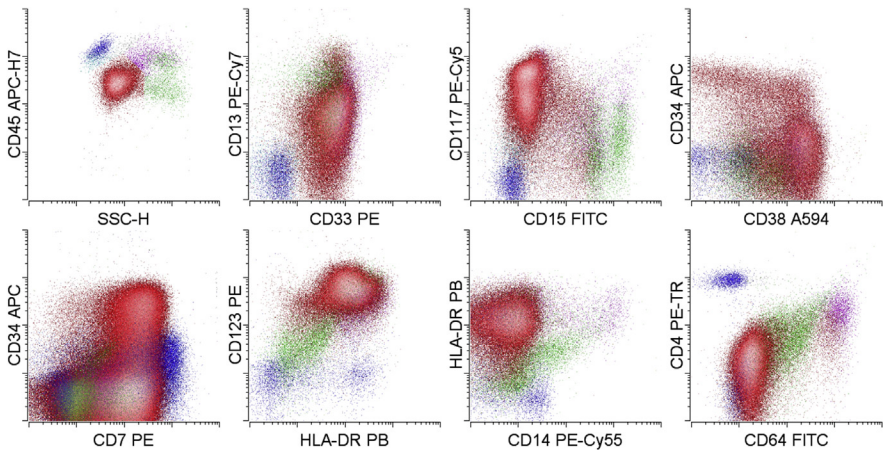

Fig. 5. Acute myeloid leukemia with FLT3 internal tandem duplication and NPM1 mutation. The leukemic blasts (*colored red*; 85.1% of the white cells) in the bone marrow show expression of CD7, CD13, CD33 (decreased), CD34 (variably decreased to absent), CD38 (variably decreased), CD117 (heterogeneous), HLA-DR (mildly decreased), and CD123 (increased) without CD4, CD14, or CD64. Some immunophenotypic features overlap with APL, although the SSC is lower than is typical for APL and in this case, the level of HLA-DR is higher than is typical for APL. Background reactive lymphoid cells are shown in *blue*, monocytic cells are shown in *pink*, and neutrophilic cells are shown in *green*.

Historically, MPAL includes acute bilineal leukemia, in which two distinct blast populations of different lineages are present, and acute biphenotypic leukemia, in which a single blast population coexpressing markers of more than one lineage is present. The diagnosis of MPAL is established in one of the three ways.[1,56] First, when two or more distinct blast populations are present, one of which would meet immunophenotypic criteria for AML and the other meet criteria for lymphoblastic leukemia (bilineal leukemia) (**Fig. 6**A). Second, a single blast population is present that meets criteria for B lymphoblastic leukemia or T lymphoblastic leukemia with coexpression of MPO (biphenotypic leukemia) (see **Fig. 6**B). Third, a single blast population is present that

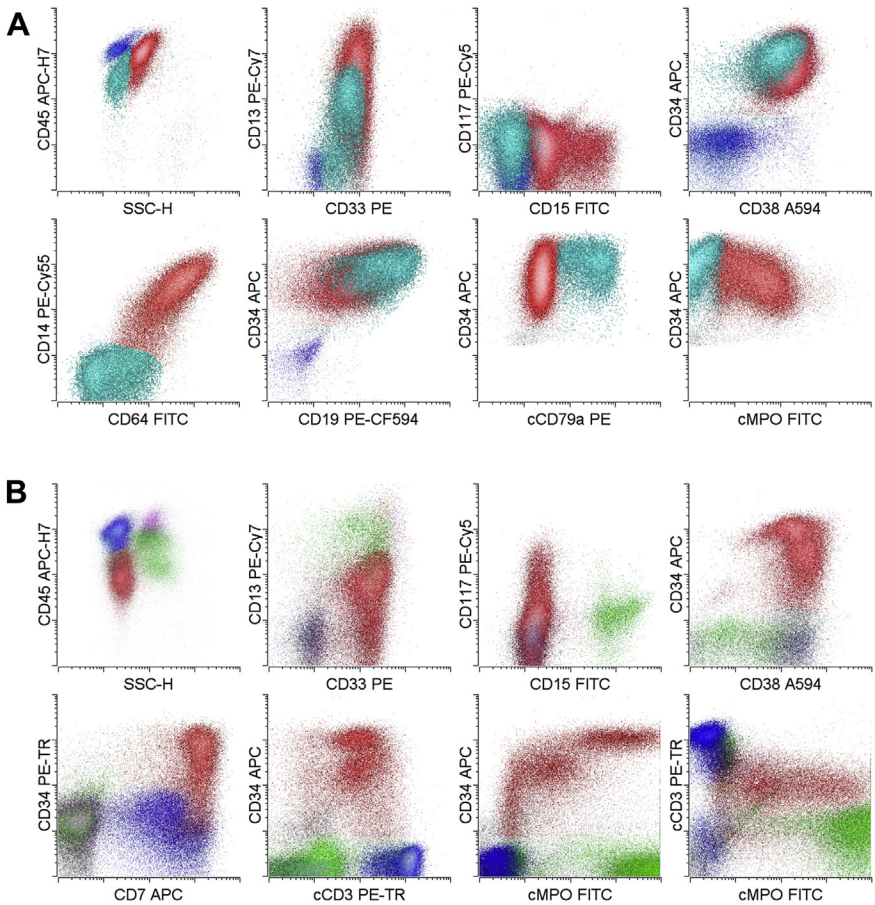

Fig. 6. Mixed phenotype acute leukemia. (*A*) B/myeloid, bilineal. The leukemic blasts in the peripheral blood are uniformly positive for CD34 and include two subsets. The dominant subset (*colored red*; 67% of the white cells) expresses CD13, CD14, CD15 (low, variable), CD19, CD33, CD34, and MPO without cCD79a or CD117. The minor subset (*colored aqua*; 10% of the white cells) expresses CD19, CD33, CD34, and cCD79a without CD13, CD14, CD15, CD64, CD117, or MPO. Background reactive lymphoid cells are shown in *blue*. (*B*) T/myeloid, biphenotypic. The leukemic blasts (*colored red*; 32.1% of the white cells) in the peripheral blood show expression of cCD3, CD7, CD13 (dim), CD33, CD34, CD38, CD117 (dim), and MPO (variable) without CD15. Background reactive lymphoid cells are shown in *blue*, monocytic cells are shown in *pink*, and neutrophilic cells are shown in *green*.

meets criteria for B lymphoblastic leukemia or T lymphoblastic leukemia and also shows evidence of monocytic differentiation (biphenotypic leukemia).

The WHO provides strict criteria for defining lineage in scenarios when a single blast population expressing markers of more than one lineage is identified. In this setting, the 2008 WHO classification and the 2016 Revision define myeloid lineage by expression of MPO or demonstration of monocytic differentiation (at least two of the following: nonspecific esterase, CD11c, CD14, CD64, lysozyme). T lineage is established by expression of strong cytoplasmic CD3 or surface CD3. B lineage assignment requires either strong expression of CD19 with strong expression of one additional B-cell marker (CD79a, cytoplasmic CD22, CD10), or weak expression of CD19 with strong expression of two additional B-cell markers. It is noted that to be considered strong, expression of CD19 or CD3 should be approaching that of a normal mature B or T cell, respectively. However, beyond that, no specific cutoff of aberrant blasts of a particular lineage is required by the WHO classification. It should be emphasized that these criteria only apply when one is considering a diagnosis of MPAL with a single blast population expressing markers of more than one lineage. These strict criteria need not be met when two distinct blast populations are identified that would each independently meet criteria for AML and acute lymphoblastic leukemia or in routine lineage assignment when making a diagnosis of a standard AML or acute lymphoblastic leukemia.

MPAL may be associated with recurrent cytogenetic abnormalities. Specifically, MPAL is further subdivided into MPAL with t(9;22); *BCR-ABL1* and MPAL with t(v;11q23); *KMT2A* (*MLL*) rearranged.[1,55,56] Most MPAL with t(9;22) have a B lymphoblast and a myeloid blast population. This category should not be diagnosed in patients with a known history of chronic myelogenous leukemia. MPAL with t(v;11q23) often contains a B lymphoblast and a monoblast population. The lymphoblast population generally expresses CD19 and is typically positive for CD15 without CD10.

Given that MPAL carries a worse prognosis than other acute leukemia subtypes, the accurate diagnosis of MPAL is critical and requires careful evaluation of available clinical, immunophenotypic, and genetic data. AML can frequently show aberrant nonlineage marker expression and some subtypes of AML characteristically show expression of nonlineage markers (eg, AML with t[8;21]). A diagnosis of MPAL should not be made in cases not meeting the strict definition as laid out by the WHO and should not be made if a different AML subcategory applies (eg, AML with recurrent genetic abnormalities, therapy-related myeloid neoplasms, AML with myelodysplasia-related changes).

Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare form of hematopoietic neoplasm derived from the precursors of plasmacytoid dendritic cells. In the 2008 WHO classification, BPDCN is classified as a subtype of AML and related precursor neoplasms,[1] whereas the 2016 Revision defines BPDCN as a separate entity from AML.[18] BPDCN frequently involves skin and bone marrow with a leukemic dissemination and has an aggressive clinical course.[1,58–60]

By flow cytometry, BPDCN typically resides in the CD45 versus SSC defined blast gate (**Fig. 7**). Neoplastic cells express CD4, CD43, and CD56 with bright CD123 and HLA-DR. Expression of CD2, CD7, CD33, CD36, CD38, and TdT may be present in a subset of cases, but CD34 and other lineage-specific and associated markers are negative including CD3, CD5, CD19, CD20, CD13, CD14, CD16, CD64, and MPO. By immunohistochemistry, BPDCN expresses TCL-1 and BDCA-2 without lysozyme. The differentiation of BPDCN from AML with monocytic differentiation is challenging

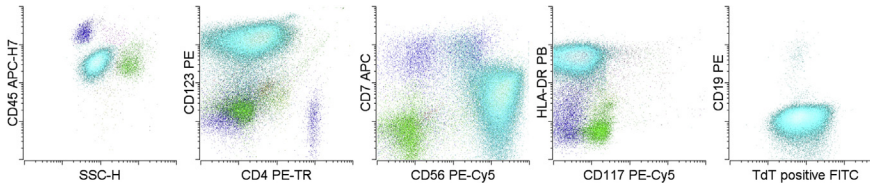

Fig. 7. Blastic plasmacytoid dendritic cell neoplasm. Flow cytometry reveals an expanded population of cells (*colored aqua*; 82% of the white cells) in the bone marrow with low CD45 and intermediate SSC. This population expresses CD4 (dim), CD7 (dim), CD56 (bright), CD123 (bright), HLA-DR, and TdT without CD34, CD117, or other B-cell or T-cell markers. Background reactive lymphoid cells are shown in *blue*, and neutrophilic cells are shown in *green*.

because both entities may express CD4, CD56, CD123, and HLA-DR. AML with monocytic differentiation typically has lower levels of CD123 and higher levels of CD64 by flow cytometry, and demonstrates expression of CD68 and lysozyme but lacks TCL-1 expression by immunohistochemistry.

DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

Minimal residual disease (MRD) in acute leukemia is defined as the presence of leukemic blasts at a level lower than the limit of conventional morphologic detection. There is growing evidence that the presence of MRD after induction therapy is independently associated with an increased risk of relapse in AML.[61–69] Given the prognostic value of MRD, sensitive, accurate, and standardized methods are required for MRD detection and monitoring; however, the clinically relevant sensitivity of testing and optimal timing for MRD assessment vary in different patient populations and in different therapeutic protocols. In the United States, multiparametric flow cytometry is the most commonly used method to evaluate MRD in AML because of its assay availability, relative affordability, and rapid turnaround time. In greater than 95% of cases of AML, leukemic blasts have an aberrant immunophenotype that can be detected by a standard antibody panel with a sensitivity of 0.1% to 0.01%.[70]

The main challenge in performing MRD detection by flow cytometry is the lack of reproducibility across laboratories caused by considerable variability in instrumentation, reagents, data analysis, and reporting. Flow cytometry also has the disadvantage of requiring a high level of expertise for data interpretation. Therefore, in order for flow cytometry for AML MRD assessment to be widely implemented, interlaboratory standardization and demonstration of reproducibility is required.

Discriminating leukemic blasts from normal hematopoietic progenitors relies on the principle that the antigen expression patterns on the leukemic blasts differ from that seen on normal cells of a similar lineage and maturation stage.[71] At present, this fundamental principle is applied in two related methodologic approaches for detecting MRD by flow cytometry.

The first approach is based on identification of a combination of aberrant antigenic expression patterns on the leukemic blasts designated “leukemia-associated immunophenotypes” (LAIPs) that are absent in normal progenitors.[72–74] At diagnosis, an antibody panel is used to define LAIPs, or regions in multiparametric space that contain only leukemic blasts but not normal progenitors of the relevant lineage. After treatment, the informative antibody panel identified at diagnosis is used on subsequent samples and leukemic blasts present in the predefined LAIP regions are considered as MRD. An extensive list of LAIPs has been described[64,66,72,75] and includes

such features as dyssynchronous antigen expression, cross-lineage antigen expression, antigen overexpression/underexpression, and aberrant light scatter. To increase sensitivity and specificity of MRD detection, all the LAIPs of the leukemic blasts detected at diagnosis should be followed in subsequent samples. Recognition of specific LAIPs may be improved by adding more fluorochromes and consequently including more simultaneous markers to define a leukemic blast with higher confidence.

Despite the successful application in some studies, this strategy has some limitations. First, LAIPs are not always stable during therapy and may shift because of leukemic blast heterogeneity.[76,77] In AML, immunophenotypic changes in expression of at least one antigen between diagnosis and relapse were observed in 88% to 91% of patients.[78,79] Such immunophenotypic shifts after treatment can lead to false-negative results. Second, similar to the leukemic blasts, the immunophenotype of the background normal or regenerating precursors may also change after therapy. Consequently, the normal precursors may appear in the regions defined for abnormal blasts, resulting in a false-positive result. Lastly, this strategy is highly dependent on the diagnostic immunophenotype. Without the knowledge of LAIPs at presentation, an individualized antibody panel cannot be constructed to accurately detect MRD.

An alternative strategy knows as the "difference from normal" approach relies on the assumption that the immunophenotype of the leukemic blasts differs from the patterns of antigen expression on normal hematopoietic progenitors of similar lineage and maturation stage.[3,80] At diagnosis, this method is similar to the identification of LAIPs, but there is no requirement to define regions for LAIPs. After treatment, using immunophenotypic aberrancies seen in the diagnostic sample as a starting point, all populations are evaluated for immunophenotypic deviations from the normal maturation pattern (**Fig. 8**). This approach avoids the limitations of LAIPs caused by immunophenotypic shift. In most cases, it is possible to detect leukemic blasts even when significant immunophenotypic changes are present in the relapsed/residual disease as compared with diagnosis. Additional advantages of the "difference form normal" approach over LAIP evaluation include that MRD is assessed without a diagnostic immunophenotype, and that a standard antibody panel is implemented.[80] However, this approach does require expert knowledge of antigen expression patterns during differentiation of normal hematopoietic progenitors in states of rest and recovery post therapy making standardization and implementation more challenging.

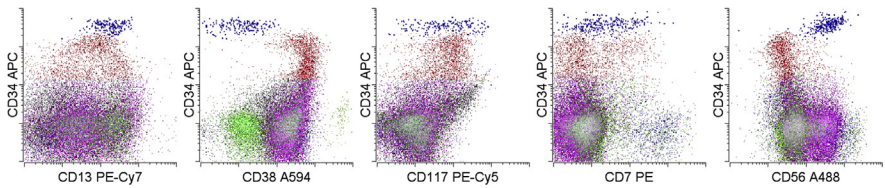

Fig. 8. The detection of MRD for acute myeloid leukemia following induction therapy by flow cytometry. The antibody combination allows the identification of residual leukemic blasts by deviation from normal hematopoietic precursors based on lineage and maturational stage. All plots show cells within the CD45 versus SSC defined blast gate. The leukemic blasts (*colored blue* and highlighted) that represent MRD are characterized by abnormal expression of CD7 (variable), CD13 (slightly increased), CD34 (increased), CD38 (decreased to absent), and CD56 as compared with the normal CD34-positive myeloid progenitors (*red*). The abnormal population is enumerated at 0.1% of the white cells. Background monocytes are shown in *pink*, and neutrophilic cells are shown in *green*.

In practice, components of both methods are commonly used simultaneously to a various degree to improve diagnostic accuracy. AML MRD assay sensitivity depends on the degree of immunophenotypic deviation of the leukemic blasts from normal progenitors, the number of normal progenitors of similar type, and the number of events acquired in each assay. The number of events recommended for acquisition and number of events to define a leukemic blast population vary significantly among groups and laboratories. In general, a sensitivity of 0.1% is achieved in AML for most patients with higher sensitivity possible for some aberrant immunophenotypes. The assay sensitivity can also vary at different time points of evaluation post therapy because some aberrant immunophenotypes may be challenging to differentiate from active marrow regeneration.

SUMMARY

Immunophenotyping by flow cytometry plays a critical role in the diagnosis and classification of AML allowing for blast identification, lineage assignment, and immunophenotypic characterization. Furthermore, flow cytometry can provide useful clues to underlying genetics, reliably distinguish AML from other precursor neoplasms that may be in the differential diagnosis, and provide insight to prognosis. AML MRD assessment has been demonstrated to be a particularly powerful tool in AML prognostication; however, this area also highlights the critical importance of standardization, which is needed to allow this modality to reach its full potential.

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